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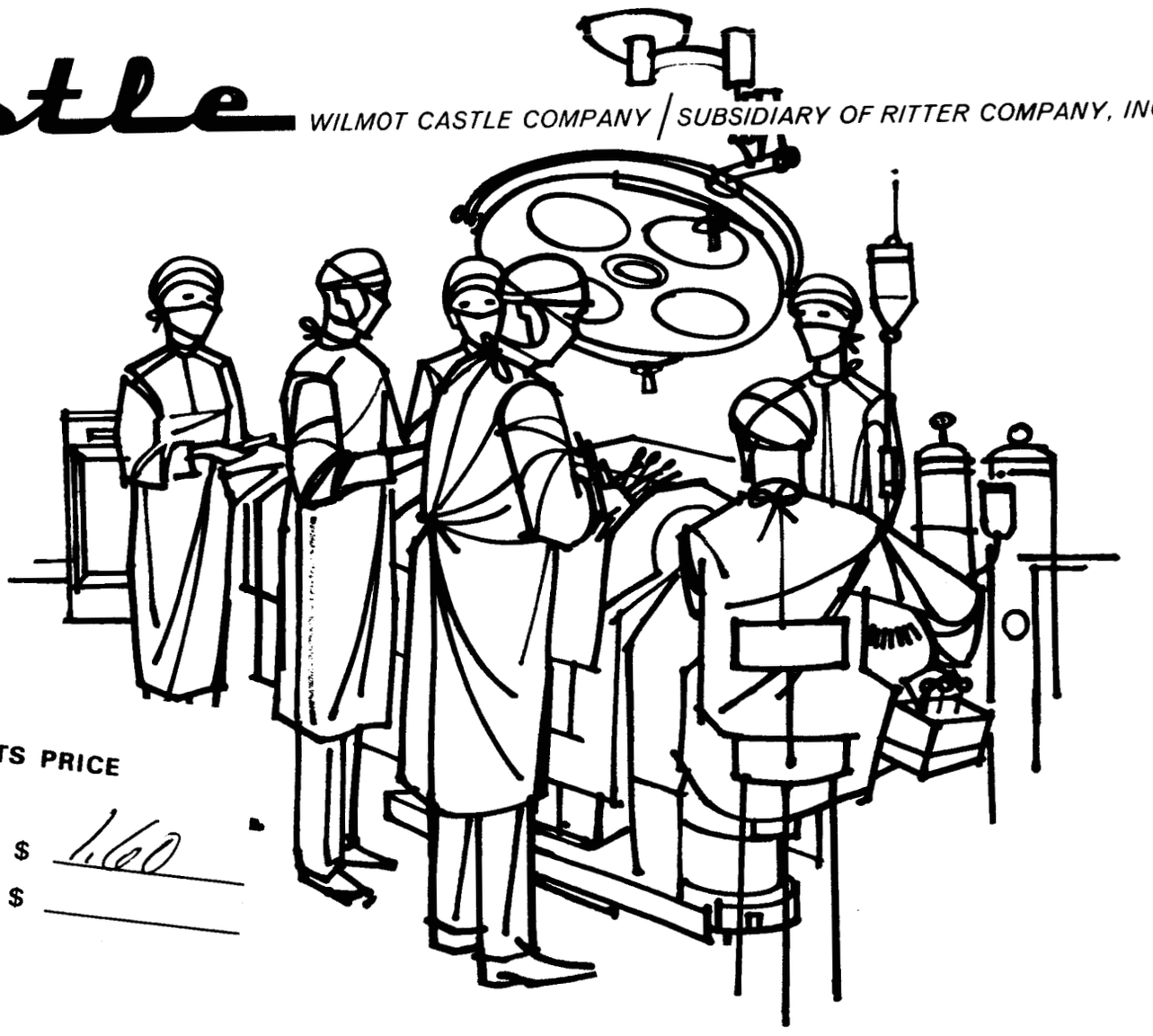

STUDIES FOR STERILIZATION

OF

SPACE PROBE COMPONENTS

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PRINCIPAL INVESTIGATOR

PROGRESS REPORT NO. 3

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

CONTRACT NASw-879

MARCH 1st, 1964 - JUNE 1st, 1964

RESEARCH LABORATORIES

WILMOT CASTLE COMPANY

ROCHESTER, NEW YORK

STATUS OF REPORTS:

This progress report covers the research performed from March 1st, 1964, to June 1st, 1964, under NASA Contract NASw-879 by the Research Laboratories of the Wilmot Castle Company.

Previous Progress Reports on this Contract were:

Progress Report No. 1 issued December 1st, 1963 and

Progress Report No. 2 issued March 1st, 1964

STATUS OF RESEARCH ACTIVITIES:

The research activities for this period have been concerned with the specific work objectives as previously outlined. These studies have been continued without any unusual problems.

CURRENT RESEARCH ACTIVITIES REPORTED ON HEREIN INCLUDE:

The current research activities which were continued or initiated during the period covered by this report include:

I. Studies on the dry heat resistance of microorganisms:

1) recovered from air samples.

a) on membrane filters.

b) on glass petri plates and microscope slides, and in jars.

c) in liquid impingers, then concentrated on membrane filters and dried.

2) added to sterile kaolin.

3) in various heated gaseous atmospheres, both non-circulating and circulating gases including air, nitrogen, and helium.

4) encapsulated in solid materials such as plaster of Paris and dental die materials.

II. Studies on components:

1) demonstration of sterility; or determination of the approximate level of contamination.

RESULTS OF CURRENT RESEARCH ACTIVITIES:

I. Studies on the dry heat resistance of microorganisms:

1) recovered from air samples:

a) on membrane filters

The sampling frequency has been increased, and the collection times have been extended to 30 days. Data is incomplete at this time.

b) on glass petri plates, on microscope slides, or in jars

Sedimentation samples on petri plates have been collected for times up to 4 months. Assay indicates levels of approximately 200 spores per plate for that time. Samples for similar time periods are being collected on slides and in jars.

c) in liquid impingers, then concentrated on membrane filters and dried

Assay of these filter pad halves have indicated that as many as 100 spores per pad have been collected in 3 hours at a sampling rate of 1 ft^3/hr.

These preliminary results are not significantly different from those reported earlier. A summary of all data obtained will be drawn up and incorporated in the final report.

I. 2) added to sterile kaolin,

Preliminary screening of the resistance of Bacillus subtilis var. niger spores in kaolin was performed on samples prepared as follows.

a) B. subtilis var. niger spores, which had been held in HCL at pH 4.5 for 1 hour were added to the sterile kaolin[1]. 0.1g samples of this preparation were not sterile after 1 hour treatment in dry heat at 135°C but were sterile after 2 hours, when cultured in trypticase soy broth.

b) B. subtilis var. niger spores which had been held in HCL at pH 4.5 for 1 hour then held in a $Ca(OH)_2$ solution at pH 10 for 1 hour were added to sterile kaolin. 0.1g samples of this preparation were not sterile at 0.5 hours treatment in dry heat but were sterile after 1 hour, when cultured in trypticase soy broth.

c) B. subtilis var. niger spores (untreated) were added to sterile kaolin. 0.1g samples were not sterile after 1 hour treatment in dry heat at 135°C but were sterile after 2 hours when cultured in trypticase soy broth.

1. The kaolin was sterilized by heating at 170°C for two 24 hour periods. Culture tests in trypticase soy broth indicated no contamination present.

It must be mentioned that at this time no attempt was made to titrate the preparations to establish their ionic nature or exchange ability. The preparations were merely treated as noted above and screened for their resistance. Assuming that such treatments did not alter the nature of the preparations, ie. alter the resistance in any way, and since no significant difference in heat resistance was found such work will be discontinued at this time. Since kaolin is reported to have a very slight ion exchange capacity it might be more rewarding to choose a material with greater capacity for any future investigations.

I. 3) in various heated gaseous atmospheres, both non-circulating and circulating gases including air, nitrogen and helium.

A comparison was made of the resistance of dry spores of B. subtilis var. niger and B. coagulans on paper strips exposed to flowing heated air and nitrogen at 125°C. The same spores were exposed in a non-flowing gaseous atmosphere in the copper-U-tube system described in progress report No. 2. Preliminary results indicate that the flowing heated gases and gases under a slight pressure (when air was replaced with the gas of choice then the closed system was heated) killed many times more organisms than when the spores exposed to the gas of choice for a similar period with equilibration to atmospheric pressure. (Tables 1 and 2)

There also appeared to be very little difference in the sporicidal effect of nitrogen as compared to air or helium on the species of dry bacterial spores employed under similar conditions.

I. 4) encapsulated in solid materials such as plaster of Paris and dental die materials:

A paste of spores of B. subtilis var. niger and B. coagulans was made using plaster of Paris, patching cement, and various dental materials. Small tablets about 3/8 inch in diameter were cast in a rubber mold. The tablets were allowed to harden and were desiccated. The residual moisture ranged from 6 to 15% by weight depending upon the compound. Each tablet weighed approximately 0.33g.

Replicate samples were treated in test tubes in the heated aluminum block. Tablets with thermocouples formed into them were also treated and the temperature recorded.

All samples are assayed for sterility by placing them in either tryptone glucose yeast extract broth for recovery of B. subtilis var. niger and trypticase soy broth for recovery of B. coagulans. The samples usually disintegrated upon standing in the broth. Any large particles remaining in the tubes after 2 weeks of incubation were broken up using a sterile glass rod, and the tubes were incubated for 2 additional weeks. Tubes giving no evidence of growth after the second incubation were inoculated with viable spores of the test organism to demonstrate lack of inhibition of the compounds.

The times for sterilization are listed in table 3. Some of these preparations are the most resistant yet found for any known organism in or on any known carrier. It takes 2 - 4 times longer to sterilize

the same levels of spores for both organisms in all of the various materials than on paper strips. There apparently is no correlation, at least at this point, between the amount of residual moisture in the various compounds and the time required to sterilize. Since the lag in heat penetration is neglible in relation to the over-all cycle (10 min or less for all temperatures employed) the increase in resistance could only result from the alteration of the physical-chemical environment of the spores.

Additional experiments are underway to determine the times, at 115, 125, and at 135°C, required to sterilize identical samples possessing other levels of the same organisms. Attempts to define and equate the time-temperature cycles will then be undertaken.

Since D values reflect differences in the resistance of organisms independent of the concentration, additional samples will be run to calculate reliable D values for each system.

II. Studies on components:

1) demonstration of sterility or determination of the approximate level of contamination:

It was previously reported that most of the commercial electronic components assayed for levels of contaminating organisms were found not to be sterile even as a result of various dry heat treatments. It was indicated in that report that very strict aseptic technique was observed. Recent evaluation of those techniques has indicated that the contamination could be actual, if re-contamination was responsible. This was confirmed prior to initiation of assays on 51 pairs of electronic components supplied by NASA. It was decided that in lieu of plate counts, the components would be broken into very small pieces with hand tools, each piece inoculated in the original tube of medium and a one ml aliquot would be withdrawn and transferred to a second tube of sterile medium <u>right in the plastic isolator</u>. This would serve as a tube dilution sequence from which an estimate of the level of viable contaminating organisms, might be made.

The controls performed in the operation of each individual isolator (ie. <u>for each individual component</u>) included:

1. Sterility tests on isolator

a) sterilization indicators - seven spore strips in glassine envelopes were placed at various sites on the plastic isolator prior to the admission of gaseous ethylene oxide

as follows:

a) 1 - on the external side of exhaust filter which is covered with tape during sterilization.

b) 4 - on interior top, bottom, front and rear walls of isolator.

c) 2 - in fingers of each glove.

b) swab sterility test on various interior surfaces of the plastic isolator.

c) contact test for sterility of glove fingers - by dipping fingers in bottle containing sterile culture broth.

2. sterility tests on incoming filtered air

a) by opening tube or bottle of sterile medium during aeration cycle.

b) by continually bubbling the air through a midget liquid impinger containing sterile broth during the testing phase.

3. sterility tests on instruments or tools were performed by dipping, rinsing, or swabbing them in sterile media both prior to and after use.

4. Surface sterility of one of the pair of components was determined by immersing it completely in a tube or bottle of sterile broth prior to assay of the interior of the second component.

5. Growth-support test on medium using a test organism to

demonstrate that no inhibition had occurred due to residual ethylene oxide. Similar inoculation tests were performed on all tubes containing broken up pieces of components after it had been established that there was no growth due to inherent contamination in or on that component. The inoculum employed was approximately 100 spores of Bacillus subtilis var. niger. Several instances of inhibition due to components or their materials have been observed.

6. Sterility test and evaluation of the component itself. The component was destroyed by employing hand tools. These pieces were then cultured in trypticase soy broth at 32°C. If no turbidity developed within two weeks, aliquots of the tube containing the original dilution were examined microscopically for microbial cells. Concurrently a subculture of the original tube was made onto TGYE agar slants and incubated for two weeks at 32°C. A sample not indicating growth in all tests was considered to have no residual viable microorganisms. All tubes were then inoculated with approximately 100 spores of B. subtilis var. niger to demonstrate the growth supporting ability of the medium. If any culture tube possessed or developed turbidity, it was subcultured onto TGYE agar slants and

a slide made to determine whether the turbidity was truly due to growth of microorganisms. Assays compiled so far on one each of 24 pairs of components and indicate that 2 (a resistor and a capacitor) were not sterile internally. The level of viable contaminating organisms was low since growth was observed in the original culture tube but not in the diluted (one ml aliquot transfer) tube. With the controls employed, it can be reliably assumed that these components were not internally sterile. Results on individual components are being tabulated and will be included in the final report upon completion of this phase.

TABLE 1

Preliminary observations on the number of dry spores of Bacillus subtilis var niger on paper strips surviving 125°C in various gaseous environments.

Gaseous Environment	Type of Unit employed	Treatment Time (hr)	Number of Survivors (organisms/strip)
		NONTURBULENT	
		0 (Control)	3.3×10^7
AIR	aluminum block	3/4	1.5×10^7
		1	5×10^6
	Copper U-tube	3/4	1.4×10^7
		TURBULENT (FLOWING)*	
		1/2	$< 10^4$
AIR	Copper U-tubing	3/4	$\sim 1 \times 10^2$
		1	$< 10^2$
NITROGEN	Copper U-tubing	1	$\sim 2 \times 10^2$

* Flow rate of gas was 2.5 CFH

TABLE 2

Preliminary observations on the number of dry spores of Bacillus coagulans on paper strips surviving 125°C in various gaseous environment.

Gaseous Environment	Type of Unit employed	Treatment Time (hr)		Number of Survivors (organisms/strip)
		NONTURBULENT		
AIR	cylindrical aluminum block	0	(Control)	6×10^6
		1/2		2×10^6
		1-1/2		1.5×10^5
	copper U-tube	1/2	(vented to atmosphere)[a]	2.6×10^6
		1/2	(sealed)[b]	2.0×10^3
		1-1/2	(vented to atmosphere)	2.6×10^4
NITROGEN	copper U-tube	1/2	(sealed)	3.5×10^3
HELIUM	copper U-tube	1/2	(sealed)	4.9×10^3
		TURBULENT (FLOWING)		
AIR	copper U-tube	1/2		4.9×10^3(c)
	copper U-tube	1/2		4.1×10^3(d)
NITROGEN	copper U-tube	1/2		5.1×10^3(c)

(a) so that no pressure would develop
(b) a slight pressure could develop upon heating the sealed unit
(c) flow of gas was 2.5 CFM
(d) flow of gas was 25 CFM

TABLE 3

Thermal death times, in hours, at three temperatures for dry bacterial spores entrapped in several solids

Compound	Time to Sterilize (Hours)							
	Bacillus subtilis var. *niger*				*Bacillus coagulans*			
	Spore Level per g*	115°C	125°C	135°C	Spore Level per g*	115°C	125°C	135°C
Plaster of Paris	1.1×10^6	28	12	6	1.3×10^5	24	14	6
Glue-base marble patching plaster	2.7×10^6	34	16	7	2.2×10^5	28	18	8
Dental Materials:								
Inlay Investment B	2.1×10^6	48	24	10	2.0×10^5	32	16	7
Bridge Model material	1.0×10^6	48	24	7	1.1×10^5	32	14	5
Paper strips	1.2×10^6	-	-	2.5	1.3×10^6		7	3

*The levels of spore contamination were found by assay of the solid materials. The weight of samples was held constant and was 0.33g for all materials. Samples solidified around thermocouples showed that all solids reached temperature in 10 min or less.

RESEARCH ACTIVITIES FOR THE FOURTH QUARTER

The research activities which will be concluded or continued during the fourth quarter of the current contract include:

1 - completing those activities currently underway as outlined in the first section of this report.

2 - Continue the long time low temperature thermal resistance studies on soil (80° to $100^{\circ}C$) initiated under the former contract (NASw-550).

3 - initiating some experiments to determine thermal cycles which would achieve sterility.

a) when based on thermal resistance values such as have been obtained.

b) when the time to reach to lethal temperatures is lengthened or exaggerated.

Report Submitted: June 1st, 1964

Martin G. Koesterer

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Principal Investigator
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Appendix - A

In addition to the activities reported herein, a summary of the thermal death time curves which could be drawn at this time based on past and current data developed in this program, was made and forwarded to Dr. Carl W. Bruch of NASA's Office of Space Sciences and Applications, Biosciences Program Division, Exobiology as per his request of June 3rd, 1964.

This summary is included in the following figure. The curves represent thermal death time values determined by the partial survival technique and are plotted here in the usual manner, ie., the longest time positives and the shortest time negatives, and then the TDT curve was obtained by drawing lines connecting these points and then the curve between them. The level of organisms upon which these curves were based:

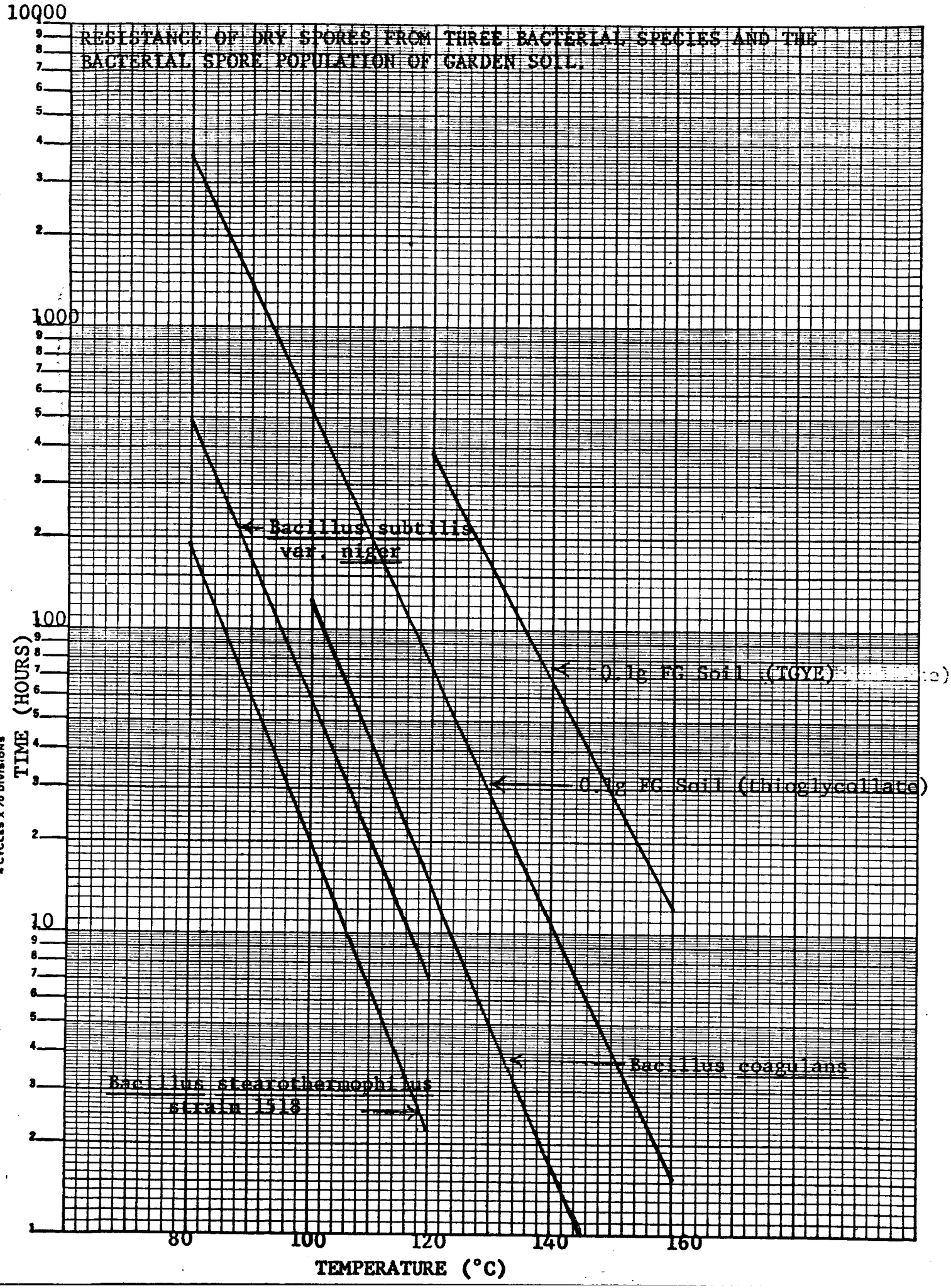
a) Bacillus subtilis var. niger — $1x10^6$ spores/paper strip

b) Bacillus stearothermophilus — $1x10^6$ spores/paper strip

c) Bacillus coagulans — $1x10^8$ spores/paper strip

d) 0.1g samples of FG soil
mesophilic aerobic spore level $3x10^5$ spores/0.1g sample

All of the pure cultures of spores were assayed for sterility in trypticase soy broth and the soil samples were assayed in thioglycollate broth and tryptone glucose yeast extract broth (TGYE). An incubation temperature of 32°C was employed for all samples except those of B. stearothermophilus and B. coagulans which were incubated at 55°C and 37°C., respectively.


RESISTANCE OF DRY SPORES FROM THREE BACTERIAL SPECIES AND THE BACTERIAL SPORE POPULATION OF GARDEN SOIL.
10000
1000
100
10
1
TIME (HOURS)
Bacillus subtilis var. niger
0.1g FG Soil (TGYE)
0.1g FG Soil (Thioglycollate)
Bacillus coagulans
Bacillus stearothermophilus strain 1518
80
100
120
140
160
TEMPERATURE (°C)